



# STUDY OF ANTISENSE OLIGONUCLEOTIDE PHOSPHOROTHIATES CONTAINING SEGMENTS OF OLIGODEOXYNUCLEOTIDES AND 2'-O-METHYLOLIGORIBONUCLEOTIDES

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**Abstract:** Antisense oligonucleotide phosphorothioates have been designed, which contain segments of oligodeoxynucleotide and 2'-O-methyloligoribonucleotides and studied for their biophysical and biochemical properties. Oligonucleotide phosphorothioates containing segments of 2'-O-methyloligoribonucleotides at both 3'- and 5'-ends show increased nuclease resistance, bind more strongly to complementary RNA targets, activate RNase H and show increased inhibition of human immunodeficiency virus type 1 replication in infected cells.

Antisense oligonucleotides and modified analogs are being widely used to modulate the expression of specific genes<sup>1</sup>. One of the most studied analogs of oligodeoxynucleotides are phosphorothioates (PS-oligonucleotide)<sup>2,3</sup>. PS-oligonucleotides are more resistant to nuclease digestion compared to their phosphodiester oligonucleotides and their duplex with RNA is a substrate for RNase H. Oligodeoxynucleotide phosphorothioates have been used as antiviral agents,<sup>4</sup> specifically inhibiting replication of human immunodeficiency virus,<sup>5-10</sup> influenza virus,<sup>11</sup> herpes simplex virus<sup>12</sup> and human papillomavirus<sup>13</sup>. Oligodeoxynucleotide phosphorothioates have also been studied for modulating expression of genes implicated in cancers<sup>14</sup> and malaria parasites<sup>15</sup>. PS-oligonucleotides have been studied for their toxicity and pharmacokinetics in mice,<sup>16</sup> rats<sup>17-19</sup> and monkeys<sup>20</sup>. PS-oligonucleotides showed animal species related side effects<sup>20</sup> and found to be degraded *in vivo* primarily by 3'-exonucleases<sup>16</sup>.

Phosphorothioate analogs of oligoribonucleotides<sup>21</sup> and 2'-O-methyloligoribonucleotides<sup>22</sup> have also been inhibitors of HIV replication, however they are less effective than PS-oligonucleotides. 2'-O-methyloligoribonucleotide phosphorothioates (PS-2'-O-methyloligoribonucleotides) are more resistant to nucleases<sup>22</sup> and form stable duplexes with RNA. However, the duplexes with RNA are not substrates for RNase H<sup>22</sup>. The present study combines the favorable antisense properties of PS-oligonucleotides (RNase H activation) and PS-2'-O-methyloligoribonucleotides (increased nuclease resistance and duplex stability) together to further improve the antisense activity of oligonucleotide phosphorothioates.

The oligonucleotide sequence used to carry out this study was complementary to the splice donor site of the tat gene of HIV-1. Various oligonucleotides (1 to 6) were synthesized (Fig. 1) which include PS-oligonucleotides, PS-2'-O-methyloligoribonucleotide and PS-oligonucleotides containing segments of PS-2'-O-methyloligoribonucleotides.

Oligonucleotides 1, 2 and 6 were studied for their nuclease resistance using snake venom phosphodiesterase<sup>23</sup>. Oligonucleotide 1 was almost completely digested in 420 min whereas, oligonucleotide 2

was digested to approximately fifty percent (Fig. 2), and Oligonucleotide 6 remained intact. These results indicate that incorporation of segments of 2'-O-methyloligoribonucleotides increased the stability of PS-

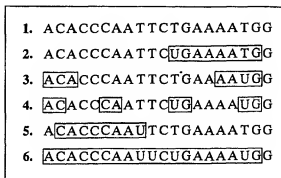


Fig. 1. Structure of the oligonucleotides synthesized. Bracketed segments of the sequence contain 2'-O-methyloligoribonucleotides.

oligonucleotide against exonucleases *in vitro* and suggest that the half-life of these oligonucleotides may improve *in vivo*. Indeed in our preliminary pharmacokinetic study in rats, PS-oligonucleotides containing 2'-O-methyloligoribonucleotides at both 3'- and 5'-ends showed a significant increase in *in vivo* half-life.

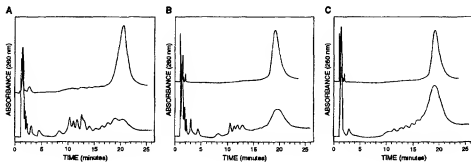


Fig. 2. Nuclease stability studies of oligonucleotide 1 (A), oligonucleotide 2 (B) and oligonucleotide 6 (C). In each block, the top HPLC profile is of zero time and the bottom HPLC profile is at 420 min.

Hybridization properties of oligonucleotide 1-6 were studied by melting temperature ( $T_m$ ) using complementary oligodeoxynucleotides as well as oligoribonucleotides to model the heteroduplex formation with a DNA and with a RNA target<sup>26</sup>. Fig. 3A shows the duplex stability studies of oligonucleotides with the complementary oligodeoxynucleotide. The  $T_m$  of the PS-oligonucleotide (oligonucleotide 1) was 51.1°C, in comparison to the  $T_m$  of the PS-2'-O-methyloligoribonucleotide (oligonucleotide 6), which was 47.6°C.

Oligonucleotides 2, 3, 4 and 5 containing various segments of PS-oligonucleotides and PS-2'-O-methyloligoribonucleotides (Fig. 1) had  $T_m$ s of 48.3, 49.9, 45.1 and 47.2°C respectively (Fig. 3A). The above results indicate that duplex stability of oligonucleotides 1 to 6 with the complementary oligodeoxynucleotide is affected adversely by incorporating segments of 2'-O-methyloligoribonucleotide phosphorothioates.

In contrast, duplexes formed between oligonucleotides 1-6 with an oligoribonucleotide showed increased melting temperatures. Fig. 3B shows the melting temperature profile of oligonucleotides 1 to 6 with the complementary oligoribonucleotide. PS-oligonucleotide (oligonucleotide 1) had a  $T_m$  of 43.4°C. In comparison, PS-2'-O-methyloligoribonucleotide (oligonucleotide 6) had a  $T_m$  of 61.1°C, which was about 17°C higher, suggesting an increased affinity of PS-2'-O-methyloligoribonucleotides to the target RNA. Oligonucleotides 2, 3, 4 and 5 had  $T_m$ s of 50.9, 48.9, 50.9 and 51.1°C, respectively. It is clearly evident from the results of this duplex stability study, that introduction of segments of 2'-O-methyloligoribonucleotide phosphorothioates into oligodeoxynucleotide phosphorothioates increases duplex stability and suggests an increased affinity to the target RNA *in vivo*.

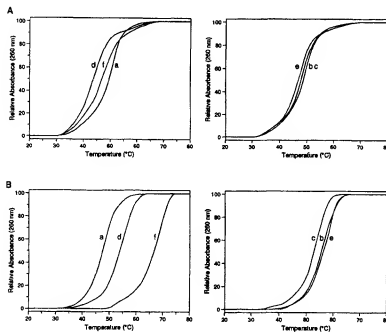


Fig. 3. Melting curves of oligonucleotide 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), and 6 (f) with the complementary (A) oligodeoxynucleotides or (B) oligoribonucleotide.

Oligonucleotide 1 (PS-oligonucleotide) and oligonucleotides 2, 3, 4 and 5, containing segments of 2'-O-methyloligoribonucleotides activated RNase H and showed site specific cleavage of the complementary oligoribonucleotide<sup>27</sup>. Oligonucleotide 6, a 2'-O-methyloligoribonucleotide, was not a substrate for RNase H and failed to show any cleavage of the complementary RNA (Fig. 4). RNase H cleavage of antisense

oligonucleotide duplexes is an important feature *in vivo* to modulate gene expression. It is achieved by heteroduplex formation which can induce cleavage of the target RNA mediated by RNase H.

Oligonucleotides were also studied for their biological activity using an acute HIV-1 infection assay<sup>28</sup>. All of the oligonucleotides inhibited virus replication, however oligonucleotides 3 and 4, containing 2'-O-methyl-oligoribonucleotide segments at the 3'- as well as the 5'-end, were more potent in inhibition of HIV-1 replication (Fig. 5). Oligonucleotide 6 showed less activity than oligonucleotide 1, even though it was more resistant to nucleases and has greater affinity for the complementary RNA but its heteroduplex with target RNA does not activate RNase H. The biological activity of oligonucleotides 1-6 confirmed the prediction based on physico-chemical properties. The most active oligonucleotides, 3 and 4, were protected against nuclease digestion from both ends, formed stable heteroduplexes with target RNA, and are able to activate site specific RNase H digestion of the target RNA.

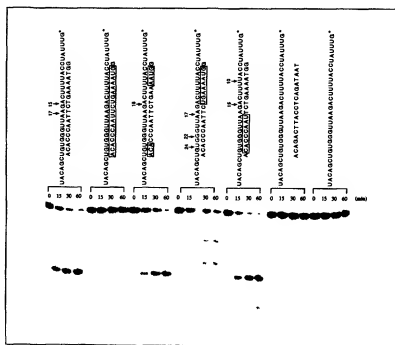


Fig. 4. RNase H activation properties of oligonucleotide. Arrows show the specific cleavage sites. Two lane at right hand sides are control RNA and RNA containing mismatched PS-oligonucleotides showed no cleavage.

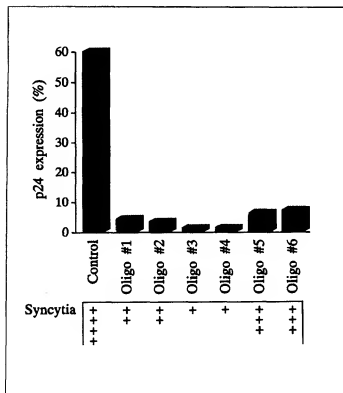


Fig. 5. Anti-HIV activity of oligonucleotides 1-6 as measured by p24 and syncytia. In syncytia assay, each + sign represents 0-25% of the cells form syncytia.

In summary, incorporation of segments of 2'-O-methyloligoribonucleotide into PS-oligonucleotides increases nuclease stability and affinity to the target RNA, without effecting the RNase H activation property. In *in vitro* experiments, these oligonucleotides are more active in inhibiting HIV-1 replication than less nuclease resistant PS-oligonucleotides or more nuclease resistant 2'-O-methyloligoribonucleotides<sup>29</sup>. A similar increase in potency of PS-oligonucleotides containing segments of 2'-O-alkyloligoribonucleotide has also been reported in a Ha-ras transfected assay system<sup>30</sup>.

#### References and Notes

1. Zamecnik, P.C. in: *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS 1991* (Wickstrom, E. ed.), pp. 1-5, Wiley-Liss, New York.
2. Stec, W.J.; Zon, G.; Egan, W.; Stec, B. *J. Am. Chem. Soc.* **1984**, 106, 6077.
3. Stein, C.A.; Cheng, Y.C. *Science* **1993**, 261, 1004.

4. Agrawal, S. *Trends Biotechnol.* **1992**, *10*, 152.
5. Agrawal, S.; Goodchild, J.; Civeira, M.P.; Thornton, A.H.; Sarin, P.S.; Zamecnik, P.C. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7079.
6. Agrawal, S.; Ikeuchi, T.; Sun, D.; Sarin, P.S.; Konopka, A.; Maizel, J.; Zamecnik, P.C. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 7790.
7. Matsukura, M.; Zon, G.; Shinzuka, K.; Robert-Guroff, M.; Shimada, T.; Stein, C.A.; Mitsuya, H.; Wong-Staal, F.; Cohen, J.S.; Broder, S. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 4244.
8. Agrawal, S.; Tang, J.Y. *Antisense Res. Dev.* **1992**, *2*, 261.
9. Lisiewicz, J.; Sun, D.; Metelev, V.; Zamecnik, P.; Gallo, R.C.; Agrawal, S. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 3860.
10. Lisiewicz, J.; Sun, D.; Weichold, F.F.; Thierry, A.R.; Lusso, P.; Tang, J.Y.; Gallo, R.C.; Agrawal, S. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 7942.
11. Leiter, J.M.E.; Agrawal, S.; Palese, P.; Zamecnik, P.C. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 3430.
12. Gao, W.Y.; Hames, R.N.; Vazquez-Padua, M.A.; Stein, C.A.; Cohen, J.S.; Cheng, Y.C. *Antimicrob. Agents Chemother.* **1990**, *34*, 808.
13. Cowsett, L.M.; Fox, M.C.; Zon, G.; Mirabelli, C.K. *Antimicrob. Agents Chemother.* **1993**, *37*, 171.
14. Ratajczak, M.Z.; Kant, A.J.; Luger, S.M.; Hijliya, M.; Zhang, J.; Zon, G.; Gewirtz, A.M. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 11823.
15. Rapaport, E.; Misiura, K.; Agrawal, S.; Zamecnik, P.C. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 8577.
16. Agrawal, S.; Tamsamani, J.; Tang, J.Y. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7595.
17. Cossum, P.A.; Sasmor, H.; Dellinger, D.; Truong, L.; Cummins, L.; Owens, S.R.; Markham, P.M.; Shea, J.P.; Crooke, S. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 1181.
18. Sands, H.; Gorey-Feret, L.J.; Cocuzza, A.J.; Hobbs, F.W.; Chidester, D.; Trainor, G.L. *Mol. Pharmacol.* **1994**, *45*, 932.
19. Agrawal, S.; Tamsamani, J.; Galbraith, W.M.; Tang, J.Y. *Clin. Pharmacokinet.* (in press).
20. Galbraith, W.M.; Hobson, W.E.; Giclas, P.C.; Schechter, P.S.; Agrawal, S. *Antisense Res. Dev.* **1994**, *4*, 201.
21. Agrawal, S.; Tang, J.Y.; Sun, D.; Sarin, P.S.; Zamecnik, P.C. in: *Antisense Strategies*, Ann. N.Y. Acad. Sci. **1992**, *600*, 1.
22. Shibahara, S.; Mukai, S.; Morisawa, H.; Nakashima, H.; Kobayashi, S.; Yamamoto, N. *Nucleic Acids Res.* **1989**, *17*, 239.
23. 0.2 A<sub>260</sub> units of oligonucleotides **1**, **2** and **6** were dissolved in 500 µl of buffer (40 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.4, 20 mM MgCl<sub>2</sub>) and treated with 0.1 U of SVPD at 37°C for 420 min. Aliquots (165 µl) of the reaction mixture were removed at 0, 200 and 420 min and analyzed on ion exchange HPLC, by using the same conditions as reported earlier<sup>24,25</sup>.
24. Metelev, V.; Agrawal, S. *Anal. Biochem.* **1992**, *200*, 342.
25. Metelev, V.; Misiura, K.; Agrawal, S. in: *Antisense Strategies*, Ann. N.Y. Acad. Sci. **1992**, *600*, 321.
26. The duplex stability study was carried out by mixing 0.2 A<sub>260</sub> units (2 µM) of oligonucleotides, listed in Fig. 1 with complementary oligodeoxynucleotide, CCAATTTTCAGAAATTGGGTGT, (20-mer) or with complementary oligoribonucleotide CCAUUUUCAGAAUUGGGUGU, (20-mer) in 500 µl of buffer (150 mM NaCl, 10 mM Na<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7). The duplex formed was heated to 85°C for 5 min and then cooled to 30°C. The temperature was then increased from 20°C to 80°C at a rate of 1°C/min and the A<sub>260</sub> was monitored with respect to temperature using Perkin Elmer Lambda 2/UV/VIS spectrometer, equipped with a Peltier thermal controller and attached to a personal computer for data accumulation.
27. The 5'-end <sup>32</sup>P labeled 32-mer synthetic oligoribonucleotide RNA (10 ng) and oligonucleotide (Fig. 1) (200 ng) were mixed in 20 µl of buffer (150 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.9) containing 1 mM DTT. The mixture was incubated with *E. coli* RNase H (6 U) at 37°C. Aliquots of 4.5 µl were removed at 0, 15, 30 and 60 min and analyzed by 20% polyacrylamide gel electrophoresis containing 7 M urea and autoradiographed.
28. MOLT-3 cells (5 × 10<sup>5</sup> per ml), cultured in RPMI medium supplemented with 15% Fetal Calf Serum were infected with HIV-1/III B. After 2 hrs of infection, cells were washed and treated with oligonucleotides at 1 µM concentration. After 4 days of incubation virus expression was measured by syncytia formation and quantitative p24 immunofluorescence assay.
29. Metelev, V. and Agrawal, S. *Proceeding of International Conference on Nucleic Acid Medical Application*, Cancun, January 1993, Abstract 1-1.
30. Monia, B.P.; Lesnik, E.A.; Gonzalez, C.; Lima, W.F.; McGee, D.; Guinasso, C.J.; Kawasaki, A.M.; Cook, P.D.; Freier, S.M. *J. Biol. Chem.* **1993**, *268*, 14514.

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